Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 170–174, January 1984
Cell Biology

Pleiotropic activities of human interferons are mediated by multiple response pathways

(prostaglandin/antiviral activity/natural killer cell activation/antigrowth activity/gene activation)

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Communicated by Sidney P. Colowick, September 9, 1983

ABSTRACT **Among the pleiotropic effects of human interferon are the inhibition of viral replication, the activation of natural killer cells, and the inhibition of cellular growth. Oxyphenbutazone, a nonsteroidal antiinflammatory agent, is a potent inhibitor of the antiviral activity of human α and β interferons as determined by cytopathic effect and vesicular stomatitis virus synthesis and release in human foreskin fibroblasts. The inhibition of interferon activity is dose dependent with maximal inhibition at 25–50 μM and minimal inhibition at 1 μM. In contrast, oxyphenbutazone at concentrations as high as 100 μM has no effect on the activation of natural killer cells by human interferon. Similarly, oxyphenbutazone has no inhibitory effect on interferon-induced antigrowth activity in the human breast carcinoma cell line MDA-MB-231. This cell line is sensitive to oxyphenbutazone inhibition of interferon-induced antiviral activity *in vitro*. In another human cell line, the vulvar carcinoma A431, oxyphenbutazone apparently augments the antigrowth activity of interferon. Although oxyphenbutazone inhibits the fatty acid cyclooxygenase enzyme in these systems, other inhibitors of cyclooxygenase fail to inactivate the antiviral activity of human interferon. Thus, oxyphenbutazone appears to inhibit the interferon antiviral cascade at a site distinct from prostaglandin biosynthesis. Moreover, the failure to inhibit natural killer cell activation or cellular antigrowth effects of human interferon suggests a pathway different from that associated with the antiviral effect of human interferon.**

Although interferon (IFN) was discovered on the basis of its antiviral activity (1–3), the IFN system has a much broader functional basis of biological activity (4, 5). Among the biological effects of the IFNs on cells and tissues of homologous origin are induction of antiviral activity, cellular antigrowth properties, and enhancement of antibody-independent natural killer (NK) cell activity in peripheral blood mononuclear cells. Using the nonsteroidal antiinflammatory agent oxyphenbutazone, we have demonstrated that the antiviral pathway of IFN-mediated activity is distinctly different from that of NK cell activation and inhibition of growth in target cells.

METHODS

The methods used are abbreviated because they have been extensively described elsewhere (6).

Cell and Virus Culture. Cells used were human foreskin fibroblasts (SG-181), a human breast cancer line (MDA-MB-231), a human vulvar carcinoma line (A431), and a NK cell target line (K562). Vesicular stomatitis virus (VSV) was propagated in HeLa cells, and titers for infectious virus were determined by plaque assay under an agar overlay.

Reagents. Oxyphenbutazone was a gift from CIBA–Geigy. Human IFN-α was produced and partially purified by the method of Familletti *et al.* (7); human IFN-β was produced and partially purified by the method of Leong and Horoszewicz (8). Titers of both IFNs were determined against INF reference standards; each had a specific activity $>10^6$ international units/mg of protein, and both were inhibited by specific antisera against each antigenic type. Culture media and fetal calf serum were obtained from GIBCO. Seaplaque agar was obtained from FMC (Rockland, ME), and tryptose phosphate was from Difco. [^{3}H]Thymidine (6.7 Ci/mmol; 1 Ci = 37 GBq) was obtained from New England Nuclear, and ^{51}Cr was obtained from ICN.

Assay of Antiviral Activity. VSV-induced viral cytopathic effect was quantitated in microtiter well plates by uptake of the vital dye, neutral red, by viable cells (6). Dye extracted from viable cells was quantitated colorimetrically at 540 nm with a Flow Titertek colorimeter interfaced with a 64K Apple II Plus microcomputer. Infectious virus release (VSV) from infected cells was quantitated by counting the number of macroscopic plaques at 5 days in the cell monolayer that developed under an agar overlay at limiting dilutions of virus.

Antigrowth Assay. The antiproliferative response to IFN was determined in tumor cells by either inhibition of [^{3}H]thymidine uptake or the inhibition of colony growth in soft agar.

NK Cell Assay. Mononuclear cells from peripheral blood of single donors were obtained by Ficoll-Isopaque gradient centrifugation. Plastic adherent monocytes were removed by incubating the peripheral blood mononuclear cells in a fetal calf serum-coated plastic culture dish for two 2-hr periods. Spontaneous natural killing activity was assayed by incubating the appropriately treated lymphocyte effector cells with ^{51}Cr-labeled K562 cells for 4 hr in RPMI 1640 medium/10% fetal bovine serum at an effector/target cell ratio of 100:1. NK cell cytotoxicity was determined as the amount of ^{51}Cr released to the medium after correction for nonspecific release.

Prostaglandin Quantitation. 6-Ketoprostaglandin $F_{1\alpha}$ and prostaglandin E_2 were quantitated in culture media by radioimmunoassay (6). The linear logit transformation of the data standards yielded a linear correlation coefficient (r) of $\geq$0.995 for all assays.

RESULTS

Oxyphenbutazone is an inhibitor of the enzyme fatty acid cyclooxygenase. We have shown previously, however, that a functional cyclooxygenase enzyme is not required for mediation of the pleiotropic activities of human IFN-α or IFN-β (6). The dose response of oxyphenbutazone inhibition of the antiviral activity of human IFN-β on human foreskin fibroblasts is shown in Fig. 1. Partial inhibition of antiviral activity is reproducible with as little as 1 μM oxyphenbutazone with 50% inhibition at 2–3 μM. Complete inhibition is achieved at 25–50 μM. In contrast, the cyclooxygenase in-

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Abbreviations: IFN, interferon; NK, natural killer; VSV, vesicular stomatitis virus.

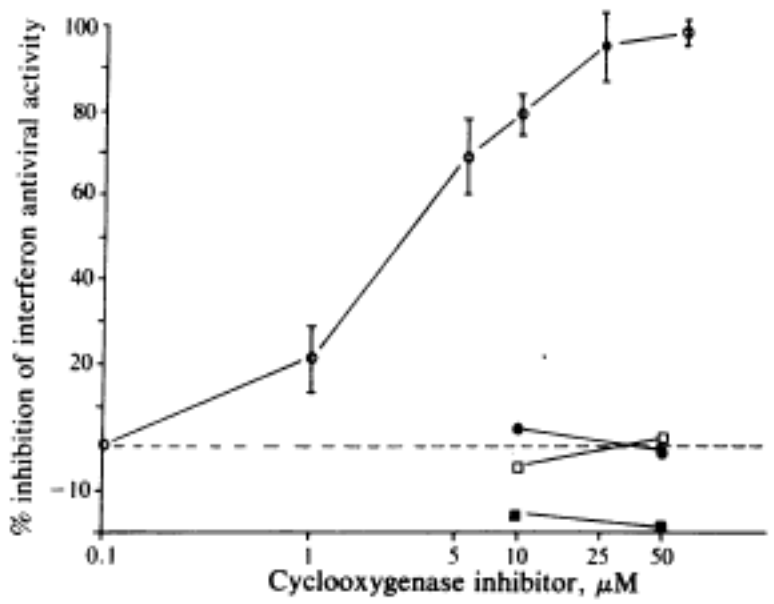

FIG. 1. Dose response of oxyphenbutazone inhibition of the IFN-induced antiviral response. Various drugs and human IFN-β (100 units/ml) were incubated in the culture medium of SG-181 cells (human foreskin fibroblast) for 2 hr. Challenge was with VSV at 0.3 plaque-forming unit per cell. IFN antiviral activity was quantitated by neutral red uptake. Results represent mean ± 1 SD. ○, Oxyphenbutazone; ●, aspirin; □, indomethacin; ■, phenbutazone.

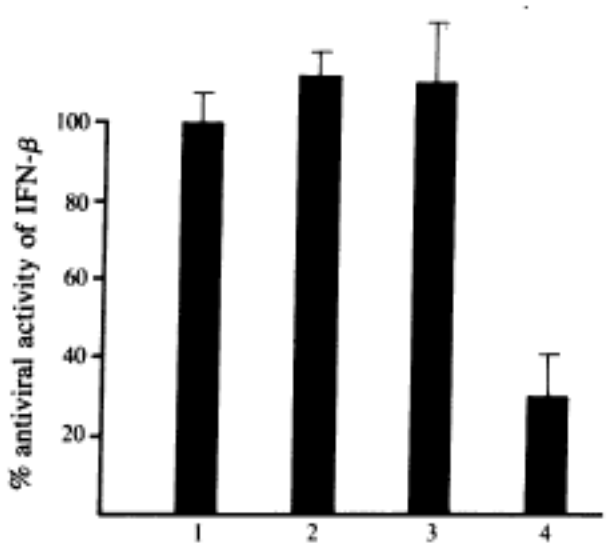

FIG. 2. Inhibition of the antiviral response in MDA-MB-231 (human breast carcinoma) cells by oxyphenbutazone. Various drugs at 50 μM and human IFN-β at 100 units/ml were added to the culture medium 2 hr prior to challenge by VSV at 0.3 plaque-forming unit per cell. IFN antiviral activity was quantitated by neutral red uptake. Results represent mean ± SD. Bars: 1, IFN; 2, IFN/aspirin; 3, IFN/indomethacin; 4, IFN/oxyphenbutazone.

hibitors aspirin, indomethacin, and phenbutazone have no effect on antiviral activity. At 50 μM, each of the cyclooxygenase inhibitors (Table 1) achieve equivalent inhibition of the cyclooxygenase-dependent synthesis of prostaglandin E_2 and 6-ketoprostaglandin $F_{1\alpha}$, the major prostaglandins elaborated by human fibroblasts (6, 9–11). Similar inhibition of the antiviral activity of IFN-β by oxyphenbutazone in a human breast cancer cell line, MDA-MB-231, is illustrated in Fig. 2. Confirmation of the inhibitory activity of oxyphenbutazone on the antiviral activity of human IFN was achieved by analysis of VSV release from infected human foreskin fibroblasts (Table 2). Although indomethacin does not inhibit infectious VSV release, 50 μM oxyphenbutazone blocks IFN-induced inhibition of virus release. Although oxyphenbutazone is an effective inhibitor of the antiviral activity of human IFN, it is apparent that its activity is not cyclooxygenase dependent.

The temporal order of addition of oxyphenbutazone and IFN to target cells is critically important to the development of the antiviral state. As shown in Table 3, oxyphenbutazone must be added prior to IFN addition to inhibit the antiviral activity of IFN. At the oxyphenbutazone concentration that reduces IFN antiviral activity by 50% when added 5 min prior to IFN, addition 5 min after IFN results in complete protection of the target cells from viral cytopathic effect. These results show that the inhibition of the IFN-induced antiviral state in target cells is an early event.

Table 1. Prostaglandin accumulation in human foreskin fibroblast (SG181) culture medium incubated with various fatty acid cyclooxygenase inhibitors

Drug	Conc., μM	Accumulation, ng/ml: 6-Keto $PGF_{1\alpha}$	Accumulation, ng/ml: PGE_2
None	—	0.92	3.51
Indomethacin	10	0.27	1.62
	50	0.24	1.15
Aspirin	10	0.27	1.87
	50	0.27	1.44
Phenbutazone	10	0.31	1.72
	50	0.25	1.19
Oxyphenbutazone	10	0.35	2.62
	50	0.20	1.36

Media were analyzed by radioimmunoassay after 18 hr of exposure to the indicated drugs together with human IFN-β (100 international units/ml) and VSV (<0.5 plaque-forming unit per cell). Quantitative determinations are based on a B/B_0 vs. log concentration of standards with a linear correlation coefficient (r) $\geq$ 0.995. Results shown are from a single experiment but are representative of several independent experiments.

Although oxyphenbutazone is an effective inhibitor of IFN-induced antiviral activity, the drug had no effect on IFN-induced NK cell activation. The enhancement of NK cell activity by human IFN on total peripheral blood mononuclear cells and lymphocytes devoid of plastic adherent monocytes and the lack of modulation of this biological response by oxyphenbutazone are shown in Fig. 3; thromboxane synthesis by contaminating platelets, however, is effectively inhibited (data not shown). Removal of the peripheral blood monocytes from the mononuclear cell population results in significant enhancement of cytotoxic activity against K562 cells, although the effector/target cell ratio remains constant (i.e., <5% monocyte removal). In unfractionated mononuclear cells, the IFN stimulation of NK cell activity is approximately 2-fold. The IFN-induced percentage increase in K562 cell cytotoxicity by monocyte depleted mononuclear cells is only 8–10% at a constant effector/target ratio.

Oxyphenbutazone has no inhibitory effect on the antigrowth properties of human IFN. The IFN-induced inhibition of [^{3}H]thymidine incorporation in the human cell lines MDA-MB-231 and A431 is shown in Fig. 4. Oxyphenbutazone, as well as aspirin and indomethacin, has no effect on the IFN-induced antigrowth activity of IFN. The IFN antigrowth effect, however, is amplified significantly in the A431 cell line while aspirin and indomethacin have no effect. To

Table 2. Effect of indomethacin and oxyphenbutazone on VSV release in human foreskin fibroblasts (SG-181)

	VSV released, pfu/ml
None	9×10^6
Human IFN-β (50 units/ml)	6×10^4
Indomethacin (50 μM)/human IFN-β	5×10^4
Oxyphenbutazone (50 μM)/human IFN-β	7×10^5

SG-181 cells were incubated for 2 hr in Eagle's minimal essential medium/5% fetal bovine serum containing the indicated drugs prior to infection with 2.8×10^4 plaque-forming units (pfu) of VSV. Cultures were incubated for 18 hr and subjected to three cycles of freeze-thaw. Replicate wells were pooled, and the growth medium was harvested for virus determination by plaque assay. The lack of indomethacin effect has been reported by Forti *et al.* (6).

Table 3. Effect of the temporal order of addition of oxyphenbutazone and IFN on VSV-induced cytopathic effect in human fibroblasts

Agent added		% protection by IFN-β	P*
At time 0	At 5 min		
IFN		100 ± 15	
OPB	IFN	49 ± 14	0.003
IFN	OPB	92 ± 15	0.434

Agents were added in the indicated order at 100 international units of human IFN-β per well and the concentration of oxyphenbutazone (OPB) that gave 50% inhibition when added prior to addition of IFN. VSV (<0.3 plaque-forming unit) was added 2 hr later, and cytopathic effect was measured by neutral red dye uptake.
*By Student's t test.

verify this observation in another analytical system, we examined the effect of oxyphenbutazone on A431 tumor colony growth in soft agar (Fig. 5). Although the apparent IFN-induced growth response is less and the variance of the mean is greater as measured by growth in soft agar, oxyphenbutazone appears to enhance the IFN-induced inhibition of A431 colony formation.

DISCUSSION

We have shown recently that a functional cyclooxygenase enzyme is not required for mediation of the antiviral, antigrowth, or NK activation effects of human IFN-α or IFN-β (6). Our results in a human system were in apparent dis-

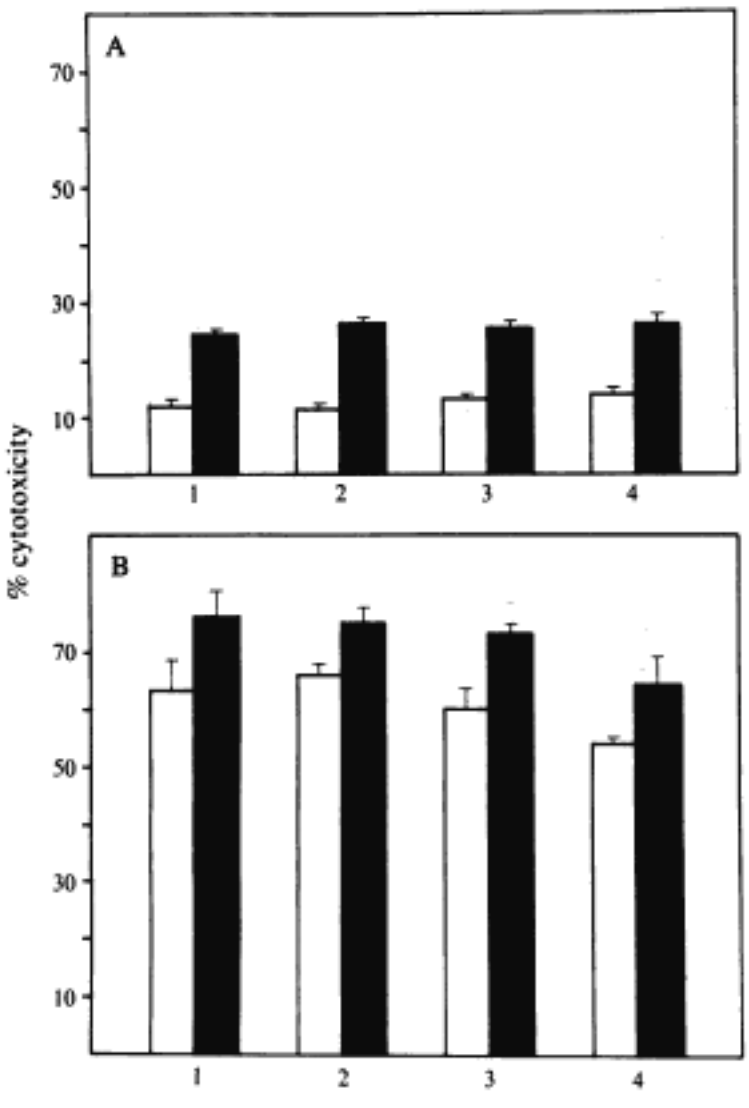

FIG. 3. NK cell activity in unfractionated (*A*) and partially purified (*B*) peripheral blood mononuclear cells in the absence (□) and presence (■) of IFN-β at 50 units/ml and various concentrations of oxyphenbutazone. (*A*) Bars: 1, no drug; 2–4, oxyphenbutazone at 1, 10, and 100 μM, respectively. (*B*) Bars: 1, no drug; 2–4, oxyphenbutazone at 1, 50, and 100 μM, respectively. Results represent mean ± SD.

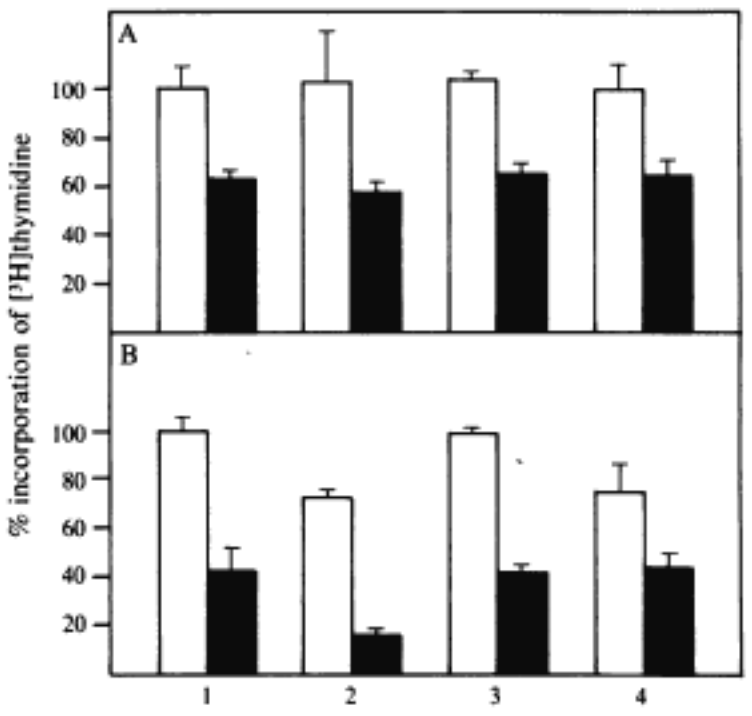

FIG. 4. Effect of oxyphenbutazone on IFN-induced antigrowth activity of MDA-MB-231 (*A*) and A431 (*B*) cells. [³H]Thymidine uptake was determined 24 hr after addition of IFN-β (100 units/ml) to culture medium. Drugs were used at 50 μM. □, No IFN; ■, IFN-β at 100 units/ml. Bars: 1, no drug; 2, oxyphenbutazone; 3, indomethacin; 4, aspirin. Results represent mean ± SD.

agreement with those of Pottathil *et al.* (12), who linked inhibition of cyclooxygenase in mouse L cells with the mediation of IFN-induced antiviral activity. Paradoxically, the addition of prostaglandin E_1, prostaglandin E_2, or 6-ketoprostaglandin $F_{1\alpha}$ also inhibited IFN antiviral activity, suggesting that the cyclooxygenase inhibitors in their murine system may be acting through mechanisms unrelated to prostaglandin biosynthesis. The same group further reported that a clone of L1210 mouse leukemia cells resistant to the antiviral and antigrowth effect of IFN had a low endogenous level of cyclooxygenase (13). However, the resistance to IFN can be more readily explained by the lack of a high-affinity receptor for IFN in the L1210 IFN-resistant clone (14). The report that inhibitors of prostaglandin biosynthesis other than oxyphenbutazone inhibit IFN induction of 2,3-dioxygenase in mouse lung (15) suggests that species differences may explain the disparity in data between mouse and human systems. It is possible that the cyclooxygenase-inhibitory drugs

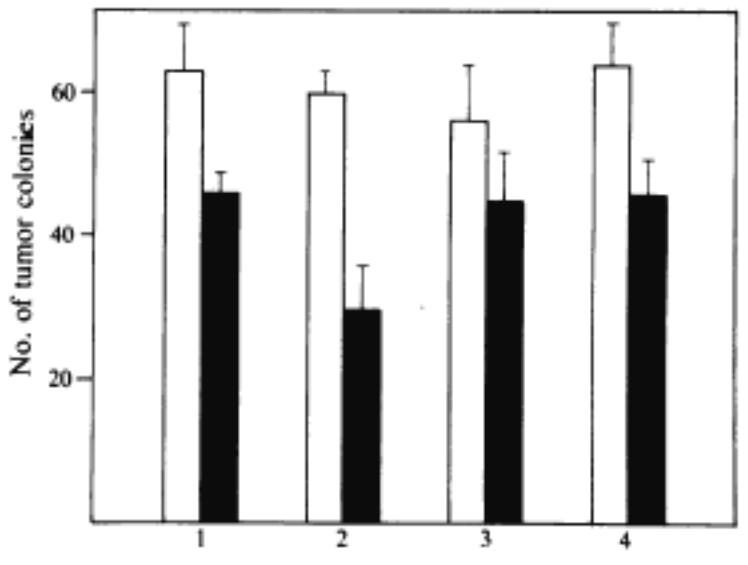

FIG. 5. Effect of oxyphenbutazone on IFN-induced antigrowth activity of A431 cells. Tumor colonies were counted 2 wk after plating in soft agar in the absence (□) or presence (■) of 100 units of IFN-β per microtiter well. Drugs were used at 50 μM. Bars: 1, no drug; 2, oxyphenbutazone; 3, indomethacin; 4, aspirin. Results represent mean ± SD.

have a wider spectrum of activity in murine systems than in man and are acting at a site distinct from cyclooxygenase but required for the mediation of IFN activity.

The differential effects of oxyphenbutazone on the mediation of the pleiotropic activities of human IFN indicate that the pathway for mediation of IFN-induced antiviral activity in target cells is different from the pathway(s) for the NK cell activation and antigrowth properties of IFN. Trivial reasons such as binding of oxyphenbutazone to IFN or to the IFN membrane receptor do not appear to be operative because differential effects in the pleiotropic activities are observed (i.e., antigrowth activity is expressed in MDA-MB-231 cells treated with IFN in the presence of oxyphenbutazone while antiviral activity is not expressed). Moreover, the conclusion that distinct molecular pathways are responsible for the differential biological effects of IFN has been suggested previously through different experiments. Evinger *et al.* (16) demonstrated that the antigrowth/antiviral activity ratios of the various species of natural human IFN-α are not constant although they are intrinsic properties of the molecules. This observation was confirmed by the demonstration that the antiproliferative/antiviral activity ratios of various recombinant human IFN-α molecules and hybrid gene IFNs varied over a 12-fold range (17). Since inhibition of IFN-induced antiviral activity by oxyphenbutazone occurs only if target cells are exposed to the drug prior to or simultaneously with IFN treatment, the oxyphenbutazone-sensitive locus must be an early step in the induction of antiviral activity. Caution needs to be exercised in the experimental use of oxyphenbutazone because, as we have observed, the drug deteriorates with age, with only 1% of the IFN-inhibitory potency remaining after 2 years of storage at room temperature (data not shown). In solution, the anti-IFN activity is greatly reduced after 1 day.

The apparent multiple pathways for IFN-induced activities may have their origin in differential gene activation. Human IFN-α and IFN-β presumably have structural homology at their active binding sites (4), since they bind to the same plasma membrane receptor (18). Little is known of the period following binding of IFN to its plasma membrane receptor (5). Following this "silent" period, there is an expression of new cellular proteins, at least four of which have specific enzymatic activities: a protein kinase (14), 2′–5′ oligoadenylate synthetase (18), a phosphodiesterase (19), and an indoleamine 2,3-dioxygenase (15). The protein kinase and 2′–5′ oligoadenylate synthetase are unique in that they require double-stranded RNA for activity. Two-dimensional gel electrophoresis using conventional staining has shown that 8–12 new proteins are induced in IFN-α- or IFN-β-treated cells (20). Since at least two of these proteins are retained on double-stranded RNA-agarose affinity columns, they may be related to the IFN-induced double-stranded RNA-dependent enzymes (21). Although the remaining new cellular proteins observed by two-dimensional gel electrophoresis may represent modification of preexisting cellular proteins, it is probable that a substantial number represented other newly induced IFN-dependent gene products. The function and significance of the other proteins and their role in IFN-induced antiviral, antigrowth, and NK activation have not been determined. However, biochemical alterations other than those cited above have been reported that probably have a direct or indirect relationship to IFN-induced gene activation. These include depression of the cytochrome P-450 system (22), stimulation of tRNA methylation (23), enhanced expression of HLA antigens including β_2-microglobulin (24), decreased membrane fluidity (25), increased actin filament polymerization (26), and enhanced expression of carcinoembryonic antigen (26) and fibronectin (27). At the most recent (1982) International Congress for Interferon Research, other biochemical alterations reported included diminished expression of $pp60^{SRC}$ in Rous sarcoma virus-transformed rat cells, expression of a myxovirus-resistance protein in mouse cells, and synthesis of a guanylate binding protein (see ref. 5 for specific citations). The multiplicity of gene products and biochemical events influenced by IFN is consistent with our observation that IFN-induced antiviral activity does not operate via the same biochemical pathway as IFN-induced NK cell activation or antigrowth effects on target cells. The dissociation of antigrowth, antiviral, and NK cell activities of IFN suggests that distinct molecular pathways are responsible for the pleiotropic biologic effects of IFN and that differential gene activation may be the mechanism by which these pathways are activated.

Although the chemical nature of the postulated mediators of IFN differential gene activation is unknown, analysis of the effect of oxyphenbutazone on cellular enzymatic processes other than cyclooxygenase-dependent prostaglandin synthesis appears to offer a useful analytical tool. It is likely that the proposed mediators are closely related in structure and that oxyphenbutazone manifests differential effects on similar enzymes using a common substrate(s). Indeed, the enhancement by oxyphenbutazone of IFN-induced antigrowth activity in A431 cells suggests just such a mechanism via substrate shunting toward an antigrowth state at the expense of a cellular antiviral state. It has been shown that the antigrowth activity of IFN cannot be predicted from its antiviral activity (16, 17). Our experience is similar (unpublished data). Thus the biological responses observed in different cells and tissues to IFN may reflect the endogenous inducible activities or partitioning of the various cellular enzymatic processes responsible for mediation of differential gene activation signals (or both).

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